

AN GHEKIERE

STUDY OF INVERTEBRATE-SPECIFIC EFFECTS OF ENDOCRINE DISRUPTING
CHEMICALS IN THE ESTUARINE MYSID *NEOMYSIS INTEGER* (LEACH, 1814)

Thesis submitted in fulfillment of the requirements
For the degree of Doctor (PhD) in Applied Biological Sciences

Dutch translation of the title:

Studie van invertebraat-specifieke effecten van endocrien-verstorende stoffen in de estuariene
aasgarnaal *Neomysis integer* (Leach, 1814)

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CHAPTER 7

**EFFECT OF NONYLPHENOL AND ESTRONE ON
MARSUPIAL DEVELOPMENT OF *NEOMYSIS INTEGER***

EFFECT OF NONYLPHENOL AND ESTRONE ON MARSUPIAL DEVELOPMENT OF *NEOMYSIS INTEGER*

ABSTRACT-----

Recently, marsupial development of *Neomysis integer* (Crustacea: Mysidacea) was described in detail by Fockedey et al. (2005a). Subsequently, we reported on the use of mysid marsupial development as a sensitive assay to evaluate endocrine disruption by exposure to the juvenile hormone analog methoprene. The present study further evaluates the potential use of this assay by exposing mysid embryos to the alkylphenol degradation product nonylphenol and the natural hormone estrone. Stage I embryos were exposed to 0.01, 1, and 100 µg nonylphenol, and 10, 100, and 1000 ng/l estrone, until hatching to a free-living juvenile. Duration of the different developmental stages, survival and hatching succes were recorded. Nonylphenol exposure had no effect on the duration of the different developmental stages but did significantly reduce survival and hatching at the highest test concentration. Estrone affected hatching at the highest exposure concentration, but had no effects on embryonic development at lower concentrations.

7.1 INTRODUCTION

Embryonic development is generally regarded as a sensitive time window for toxic effects during an organism's life history. *Neomysis integer*, like other mysids, carries its embryos in a marsupium where the entire larval development takes place from oviposition to the release of free-living juveniles (Wittmann, 1984). The marsupial development of *N. integer* was recently described in detail by Fockedey et al. (2005a). Marsupial development in mysids was subsequently shown to be a sensitive endpoint to evaluate the effects of the insecticide,

methoprene (Chapter 6). Our ongoing and future efforts are to further explore mysid early embryonic development as a sensitive target to assess endocrine disruption.

ENDIS-RISKS is a multidisciplinary project that has studied the occurrence, distribution and potential effects of endocrine disruptors on mysids in the Scheldt estuary (Belgium/The Netherlands), one of the most polluted estuaries in the world (ENDIS-RISKS project, <http://www.vliz.be/projects/endis>). We earlier reported high levels of organotins, surfactants and flame retardants in mysids of the Scheldt estuary (Verslycke et al., 2005). More recent studies found significant levels of estrone in water of the Scheldt estuary (Noppe et al., 2005). The effect of nonylphenol and estrone on the vitellogenesis of *N. integer* are described in more detail in Chapter 4 (§ 4.1). This chapter describes the effects of nonylphenol and estrone on marsupial development of *N. integer*.

7.2 MATERIAL AND METHODS

7.2.1. Chemicals

Estrone was obtained from Sigma-Aldrich (Bornem, Belgium) and nonylphenol from Acros Organics (Geel, Belgium). Stock solutions of the test compounds were prepared in absolute ethanol. The ethanol concentration in the solvent control and in the different test concentrations was 0.01%.

7.2.2. Test organisms

Mysid crustaceans, *Neomysis integer*, were collected in March 2005 by handnet in the Braakman, a brackish water (10 psu) near the Scheldt estuary in Hoek (The Netherlands). Mysids were taken to the laboratory and cultured as described in Chapter 2 (§ 2.2.1.).

7.2.3. Test design

A detailed description of the marsupial development assay in *N. integer* is described in Chapter 6 (§ 6.2.3). In short, stage I embryos were transferred at random to each of the wells of a 12-cell plate each containing 4 ml of the different exposure concentrations of nonylphenol and estrone (dilution water is artificial seawater of 15 psu). Embryos were exposed to 0, 0.01, 1, and 100 µg nonylphenol/l, and 0, 10, 100, and 1000 ng estrone/l. All

concentrations reported in this study are nominal, based on dilutions of the stock solutions. Fifteen replicates per concentration, and a minimum of 6 embryos per replica, were used. Multiwells containing the embryos were placed on an orbital shaker (80 rpm), and survival, developmental stage and hatching were recorded daily. Concurrently, dead embryos were removed and 75% of the medium was replaced.

The different developmental stages of the mysid embryo were distinguished as described in Chapter 6 (§ 6.2.4.). Stage I embryos are spherical and stage I ends with hatching from the egg membrane (Fig 7.1). Stage IIa embryos are dorsally bent and have a comma-like appearance. In stage IIb, the body is further extended and the thoracic appendages become more elongated. Stage II ends with molting of the embryo. Stage III embryos have stalked eyes and a developed telson and uropods. Stage III terminates with a final molt, leading to free-living juveniles.

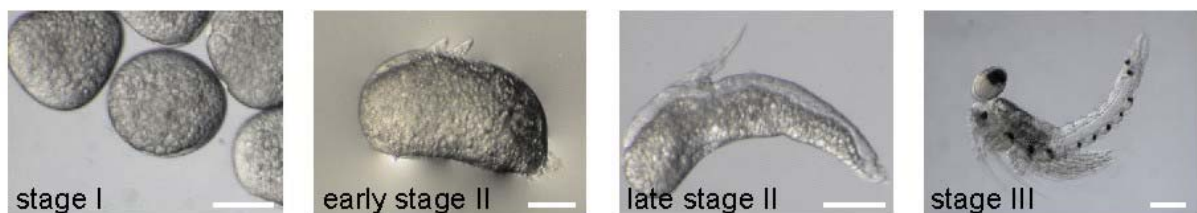


Figure 7.1: Different developmental stages of *Neomysis integer* embryo. Scale bar = 250 μm .

7.2.4. Statistics

All data were checked for normality and homogeneity of variance using Kolmogorov-Smirnov and Levene's test respectively, with an $\alpha = 0.05$. The effect of the treatment was tested for significance using a one-way analysis of variance (Dunnett's test; Statistica™, Statsoft, Tulsa, OK, USA). All box-plots were created with Statistica™ and show the mean (small square), standard error (box), and the standard deviation (whisker).

7.3. RESULTS

7.3.1. Survival

Percentage embryo survival was calculated each day for the different exposure concentrations (Fig. 7.2). Survival was highest during stage I for both chemicals. Exposure to all test concentrations of estrone had no significant effect on survival in all three different developmental stages of the embryo. Exposure to the highest concentration nonylphenol (100 µg/l), however, resulted in significant mortality on embryos of stage II and III.

7.3.2. Duration of the different developmental stages

Figure 7.3 shows the duration of the different developmental stages of the embryos exposed to nonylphenol and estrone.

Duration of stage I was approximately 3 days, while stage II and III lasted about 6 and 3.5 days, respectively. The total embryonic development time of *N. integer* embryos development was around 12.5 days. Nonylphenol and estrone had no effect on the duration of the different developmental stages and the total embryonic development time of *N. integer*.

7.3.3. Hatching

Exposure to 100 µg nonylphenol/l, and 1000 ng estrone/l resulted in significantly lower hatching rates compared to the control (Fig. 7.4). Average hatching percentages were $81.4 \pm 19.4 \%$, $78.1 \pm 14.5 \%$, $82.1 \pm 13.8 \%$ and $11.1 \pm 16.2 \%$ for the control, 0.01, 1 and 100 µg nonylphenol/l treatments, respectively. For estrone, average hatching percentages were $81.1 \pm 15.2 \%$, $75.5 \pm 19.8 \%$, $76.2 \pm 16.9 \%$ and $64.2 \pm 11.0 \%$ for the control, 10, 100, 1000 ng estrone/l treatments, respectively.

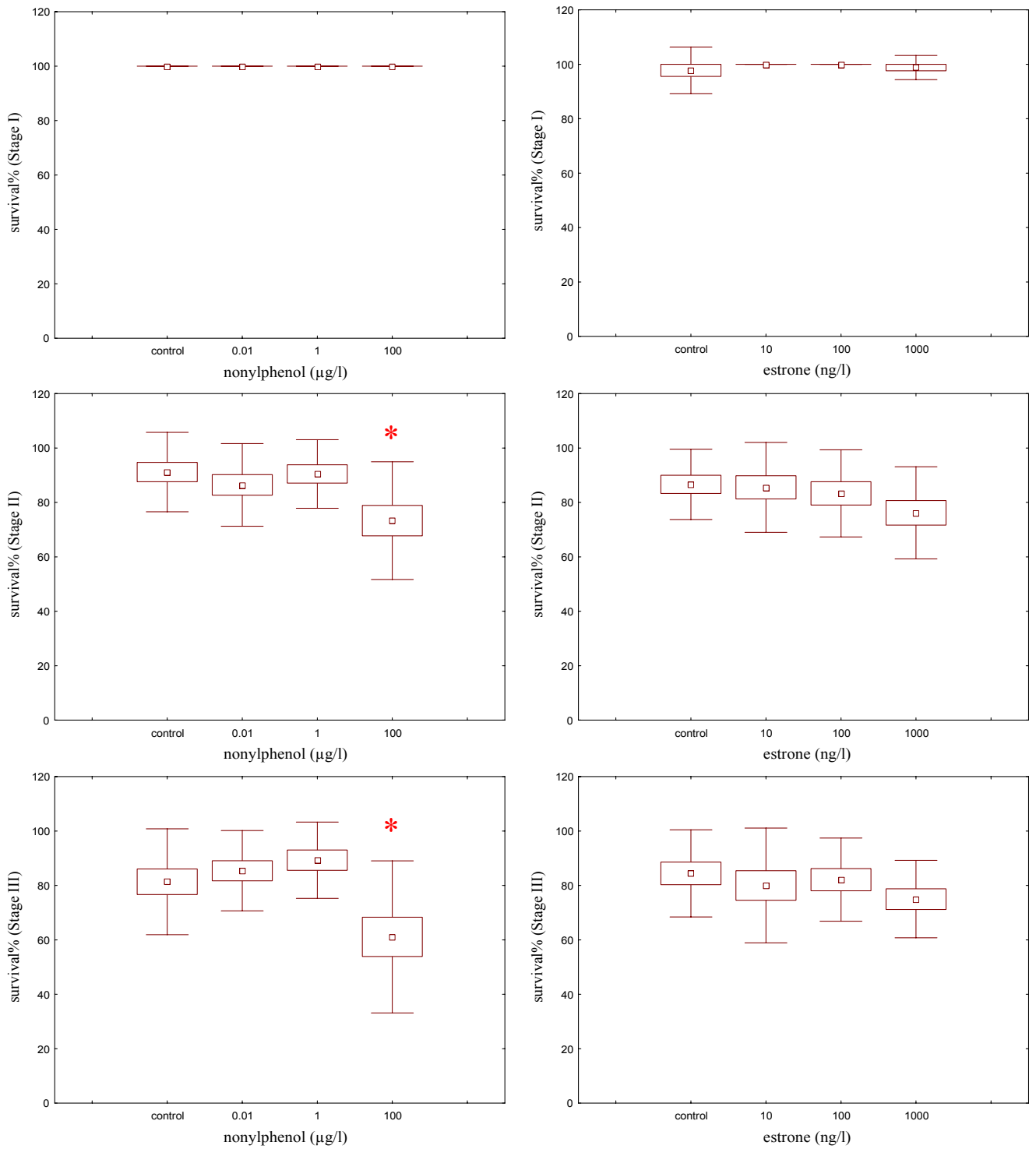


Figure 7.2: Percentage survival of *Neomysis integer* embryos during the different developmental stages exposed to 0.01, 1, 100 µg nonylphenol/l (left), and 10, 100, 1000 ng estrone/l (right). (Anova, Dunnett; *p<0.05, significance from control).

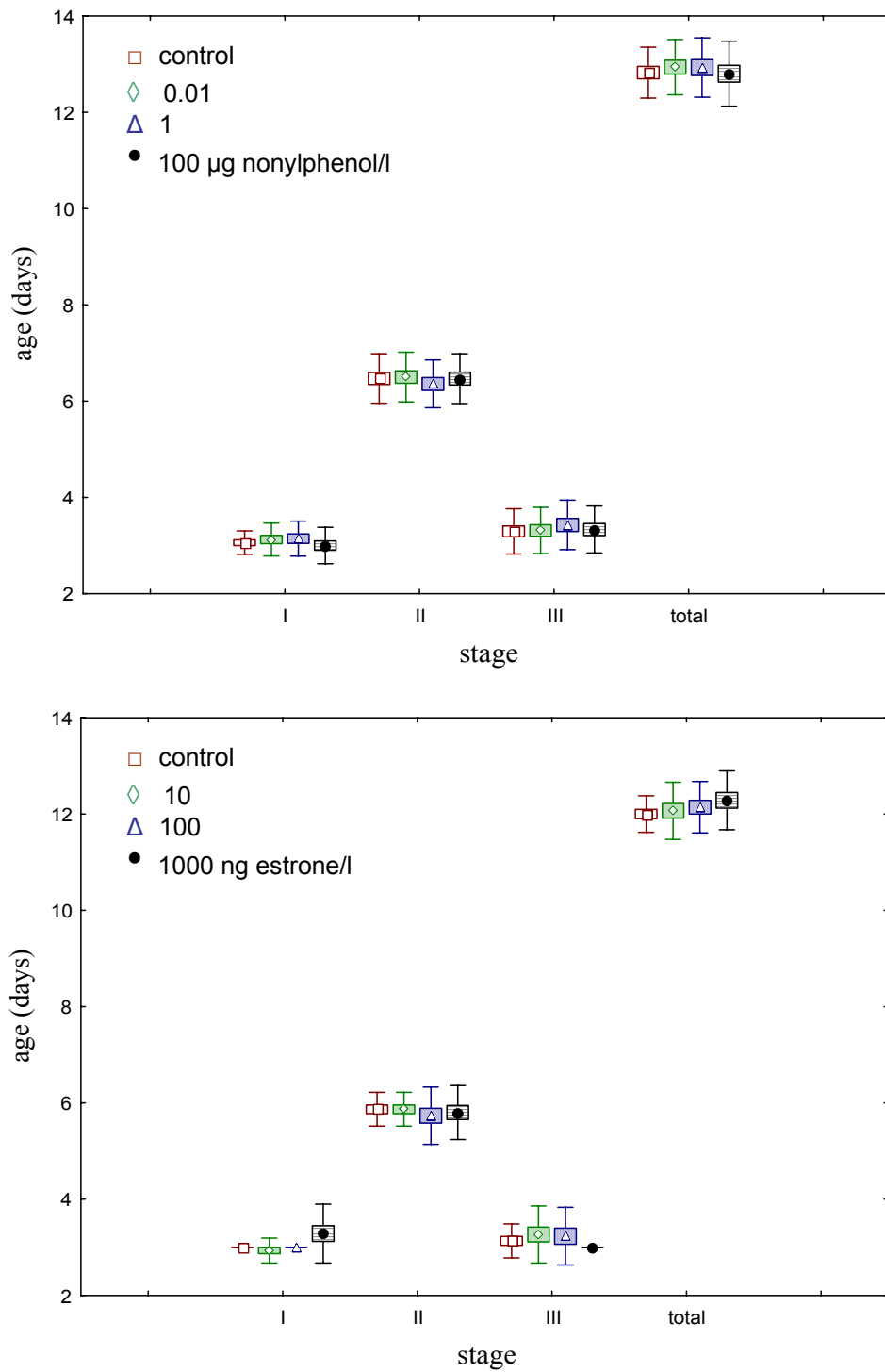


Figure 7.3: Duration of the different developmental stages of *Neomysis integer* embryos (days) exposed to 0.01, 1, 100 µg nonylphenol/l (above), and 10, 100, 1000 ng estrone/l (below). Total= duration of the total development time.

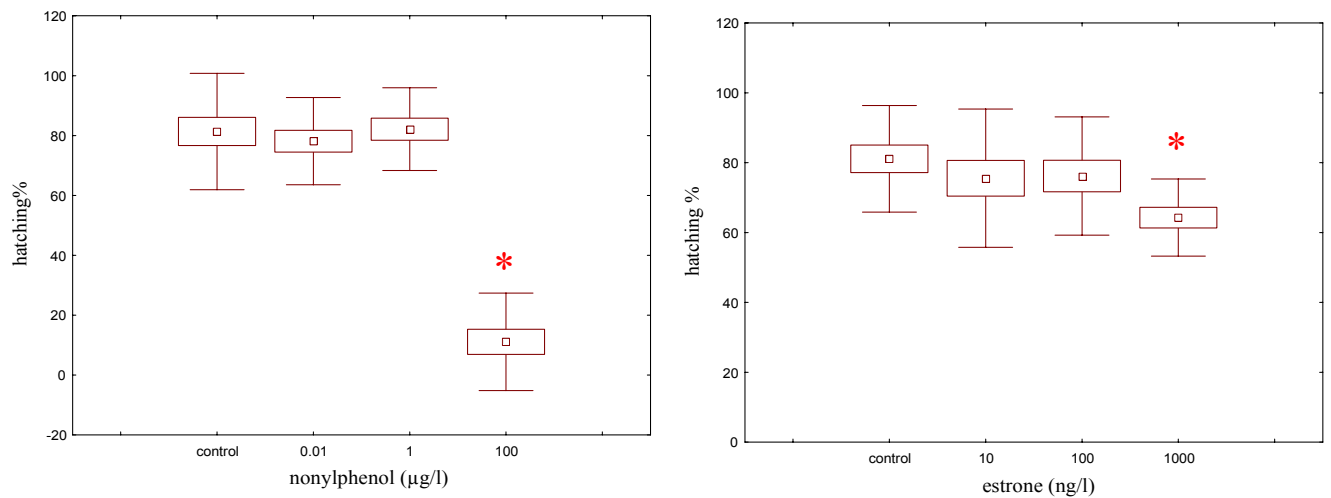


Figure 7.4: Percentage hatching of *Neomysis integer* embryos exposed to 0.01, 1, 100 µg nonylphenol/l (left), and 10, 100, 1000 ng estrone/l (right) from oviposition till free-living juveniles. (Anova, Dunnett; * $p < 0.05$, significance from control)

7.4 DISCUSSION

In this doctoral study, we described the development of three physiological processes in *Neomysis integer* that are regulated by invertebrate-specific hormones, the ecdysteroids (Chapter 1). These three processes, vitellogenesis (Chapter 4), molting (Chapter 5), and embryogenesis (Chapter 6) were subsequently evaluated as endpoints to assess endocrine disruption through exposure with the test compound methoprene. These experiments demonstrated that methoprene lowers vitellin levels in *N. integer*, but this effect was not statistically significant. Methoprene did affect mysid molting at 100 µg/l, and embryogenesis at 1 µg/l. These results indicate that embryogenesis was the most sensitive endpoint at detecting the endocrine-disruptive effects of methoprene.

In this chapter we evaluated the potential effects of nonylphenol and estrone on the embryogenesis of *N. integer*. Estrone is frequently detected in water of the Scheldt estuary at concentrations of up to 8 ng/l (Noppe et al., 2005, Vethaak et al., 2002). Nonylphenol concentrations in the Scheldt estuary are similar to levels found in U.K. rivers and are around 10 µg/l (Verslycke et al., 2005; Vethaak et al., 2002). Nonylphenol and estrone did not affect embryogenesis of *N. integer* at environmentally relevant concentrations. However,

nonylphenol did significantly reduce survival and hatching at 100 µg/l when compared to the control. Estrone significantly lowered hatching at 1000 ng/l. Estrone was previously shown to affect mysid vitellogenesis at 1000 ng/l by lowering vitellin levels in females (Chapter 4). In the same study, nonylphenol increased vitellin levels at 0.01 µg/l.

We also observed that hatched embryos exposed to 1 and 100 µg nonylphenol/l were more lethargic, and higher mortality was observed at these concentrations during the first two days after final embryonic molt to free-living juveniles. Behavioural changes will be examined in future studies and might provide a sensitive way for evaluating endocrine disruption. Forget-Leray et al. (2005) found that nonylphenol significantly reduced molting success in the copepod *Eurytemora affinis* (LOEC of 15 µg/l). Molting from one stage to another was often incomplete. Animals from unsuccessful molt were deformed and displayed a mix of naupliar and copepodid characteristics, and were also unable to move.

Alkylphenols, natural hormones and synthetic hormones, have been suggested as the most likely candidate chemicals responsible for the 'feminization' detected in male fish (Desbrow et al., 1998; Vos et al., 2000). Effects of these vertebrate (xeno-) estrogens on crustaceans, however, remains controversial and poorly understood giving the current knowledge on the presence of vertebrate-type steroids, such as estrogens, in these animals (Billingham et al., 2000; Sanders et al., 2005; Tsukimura, 2001). As such, existing studies on the effects of (xeno-)estrogens on crustacean vitellogenesis remain fragmented and contradictory (Chapter 4, § 4.4). However, the present study further adds to the weight-of-evidence that (xeno)estrogens appear to be less effective in causing endocrine disruption in crustaceans than in oviparous vertebrates. This also supports the growing consensus that invertebrate-specific approaches are needed for a relevant assessment of the risk of endocrine disrupting chemicals to invertebrates as they are most likely affected through hormonal pathways that are different from those of vertebrates.